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INHIBITION OF RAT HEPATIC MITOCHONDRIAL ALDEHYDE DEHYDROGENASE ISOZYMES BY REPEATED CYANAMIDE ADMINISTRATION: PHARMACOKINETIC–PHARMACODYNAMIC RELATIONSHIPS

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ABSTRACT

The inhibition of rat hepatic mitochondrial aldehyde dehydrogenase (ALDH) isozymes was studied in apparent steady-state conditions after repeated intra-peritoneal cyanamide administration. The low-K_m mitochondrial ALDH isozyme was more susceptible to cyanamide-induced inhibition ($DI_{50} = 0 \cdot 104$ mg kg^{-1}) than the high-K_m isozyme ($DI_{50} = 8 \cdot 52$ mg kg^{-1}), with almost complete inhibition occurring at $0 \cdot 35$ mg kg^{-1} total cyanamide administered for the low-K_m isozyme. The relationships between plasma and liver cyanamide concentrations and the inhibition of high-K_m ALDH were established by means of the sigmoid I_{max} model. The effect of dosing rate on the plasma concentration of cyanamide at apparent steady-state showed non-linearity, indicating that clearance or first-pass metabolism of cyanamide during its absorption after intra-peritoneal administration did not remain constant throughout the range of doses studied.

KEY WORDS Cyanamide Rat Liver Mitochondrial aldehyde dehydrogenase Isozymes Inhibition

INTRODUCTION

Cyanamide is commonly referred to as an alcohol deterrent agent due to the aversive effects produced during its interaction with ethanol.[1] This drug is a potent inhibitor of rat liver aldehyde dehydrogenase (ALDH) isozymes *in vivo*,[2–8] in hepatocytes,[9] mitochondria[10] or liver slices.[11] In contrast, cyanamide does not inhibit *in vitro* the purified liver ALDH from different species.[5,6,12] The inactivation *in vitro* of ALDH has been observed with partially purified enzyme preparations from sheep[13] and rat,[7] and with rat intact mitochondria

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and isolated microsomes.[14,15] These results led some authors[5,6,12,14] to hypothesize an enzyme catalysed conversion of cyanamide to an active metabolite, which would be responsible for ALDH inactivation.

Cyanamide is metabolized via two different pathways. The primary route, which represents a detoxification pathway, is catalysed by acetyl-S-CoA dependent hepatic *N*-acetyltransferase yielding *N*-acetylcyanamide, the major urinary metabolite of cyanamide.[16] However, *N*-acetylcyanamide did not inhibit ALDH *in vitro* even in the presence of the cyanamide-activating enzyme. DeMaster *et al.*[17] have shown that catalase is the enzyme responsible for the metabolic activation of cyanamide. Thus, the second pathway seems to be an activation reaction catalysed by catalase.[11,17,18] Recently, it has been pointed out that cyanamide itself could be the inhibitor of ALDH in a reaction mediated by catalase in the presence of NAD^+.[19]

Detailed information about the pharmacokinetic–pharmacodynamic ALDH inhibition relationship for cyanamide in humans and experimental animals is scarce. In this sense, Brien and Loomis[20] studied the time course of hepatic ALDH inhibition in the subcellular fractions, the pharmacokinetics of carbimide, and the relationship between plasma carbimide concentration and the inhibition of each hepatic ALDH isozyme following a single oral dose of calcium carbimide. The aim of the present study was to determine, under apparent steady-state conditions, the relationships between plasma, liver, and mitochondrial concentrations of cyanamide and the inhibition of mitochondrial ALDH isozymes for a wide range of cyanamide doses.

MATERIALS AND METHODS

Chemicals

Cyanamide was purchased from Fluka (Buchs, Switzerland), acetaldehyde from Merck (Darmstadt, Germany), NAD^+ from Boehringer Männheim (Männheim, Germany) and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO).

Experimental protocol

Male Sprague-Dawley rats, weighing 240–300 g, were anaesthetized with diethylether before blood was withdrawn by cardiac puncture. Afterwards, the rats were killed by decapitation and the livers were perfused *in situ* with ice-cold 0·25 M sucrose solution.

Cyanamide in saline solution was administered *intra*-peritoneally in seven doses (0·005, 0·010, 0·025, 0·05, 0·1, 0·5, 1, 2, 8, 16, and 32 mg kg^{-1}) at 45-min time intervals to attain steady-state conditions. The selected dose interval was near to the reported plasma elimination half-life value of this drug in the rat.[21,22] The rats were killed 45 min after the last drug dose.

Determination of ALDH activity

To measure the mitochondrial ALDH activity, the mitochondrial fraction was obtained according to the method described by Tottmar *et al.*[23] An aliquot of liver was homogenized in ice-cold 0·25 M sucrose solution (1 : 10 w/v) and the mitochondrial fraction was washed twice with the same solution. The pellet was resuspended in 0·25 M sucrose solution containing 1 per cent Triton X-100 and centrifuged at 87 000 × **g** for 1 h. The ALDH activity in the supernatant thus obtained, was measured spectrophotometrically by following the reduction of NAD^+ at 340 nm (25°). The reaction mixture contained 0·5 mM NAD^+, 0·1 mM pyrazole, 2 μM rotenone, 50 μM or 5 mM acetaldehyde and 50 mM sodium pyrophosphate buffer (pH 9·3). The reaction was started by the addition of acetaldehyde; 50 μM for low-K_m isozyme activity and 5 mM for total ALDH activity. High-K_m isozyme activity was obtained by subtracting the low-K_m ALDH activity from the total activity.

Determination of cyanamide concentration

To determine the concentration of cyanamide in the hepatic mitochondria and whole liver, appropriate mitochondrial and liver extracts were prepared as described below.

A liver sample (2 g) was homogenized (1 : 10 w/v) with sucrose solution. The homogenate was centrifuged at 480 × **g** for 10 min and the supernantant was centrifuged again at 4200 × **g** for 7 min to obtain the mitochondrial fraction. Mitochondria were resuspended in 3 ml 5 mM sodium phosphate buffer (pH 7·4) and centrifuged, after 30 min, at 30 000 × **g** for 30 min. The obtained supernatant constituted the mitochondrial extract.

Another liver sample (3 g) was homogenized (1 : 3 w/v) in 5 mM sodium phosphate buffer (pH 7·4) and centrifuged, after 30 min, at 30 000 × **g** for 30 min. The supernatant constituted the liver extract for the determination of the concentration of cyanamide.

The concentrations of cyanamide in the liver and mitochondrial extracts were determined by a method involving extraction of cyanamide from these aqueous supernatants into ethyl acetate. For this purpose, samples of 2 ml from different extracts were extracted with 4 ml ethyl acetate. Three millilitres of each of the organic phases were transferred to conical tubes and evaporated to dryness under a stream of nitrogen. The residues were dansylated and the dansyl-derivative of cyanamide was quantitated by an HPLC technique using fluorimetric detection, as has been described for the determination of cyanamide in plasma.[24] The calibration curves were constructed using data obtained with spiked samples of both liver and mitochondrial extracts obtained from untreated rats. The lower limits of quantitation for the determination of cyanamide in plasma, liver, and mitochondrial extracts were 4 ng cyanamide ml^{-1} plasma, 30 ng cyanamide g^{-1} liver and 180 ng cyanamide g^{-1} mitochondrial residue, respectively.

Protein was determined by the method of Lowry *et al.*[25] using bovine serum albumin as standard.

Data analysis

The experimental results were expressed as mean ± SEM of five animals.

DI_{50}, dose of cyanamide producing 50 per cent of the maximum inhibition (I_{max}), was estimated by regression analysis of the total dose–effect (% inhibition) curve using the non-linear regression method (MULTI program[26]). Fitting of the plasma cyanamide concentration vs dosing rate curve was performed by means of the PCNONLIN program using the reciprocal of the square standard deviation as a weighting factor. Equations (1) and (2) representing I_{max} and sigmoid I_{max} models, respectively, were fitted to the experimental data-pairs of per cent of ALDH inhibition and plasma concentration of cyanamide at apparent steady-state (C_{SS} values) by means of a least squares extended non-linear regression method using the MKMODEL program.

$$\%I = \frac{I_{max}\, C}{CI_{50} + C} \tag{1}$$

$$\%I = \frac{I_{max}\, C^n}{CI_{50}{}^n + C^n} \tag{2}$$

In these equations, $\%I$ represents the per cent inhibition of the isozyme activity, I_{max} the maximum inhibition, CI_{50} the concentration of cyanamide producing 50% of I_{max}, C the concentration of cyanamide, and n the Hill coefficient.

The selection of the model that best fitted the experimental data was performed by the application of the Schwarz criterion,[27] which takes into account the goodness of fit and the number of parameters of the model according to the following equation:

$$\text{Schwarz criterion} = \log \text{likelihood} - (K \ln(N))/2$$

K being the number of parameters and N the number of experimental data. The larger the Schwarz criterion the better is the model able to describe the data.

RESULTS

The relationship between plasma, liver, and hepatic mitochondrial cyanamide steady-state concentrations and dose of cyanamide was studied. For this purpose, seven repeated intraperitoneal administrations of cyanamide were given with a

Table 1. Relationship between mean (±SEM; $n=5$) plasma, liver and mitochondrial cyanamide concentrations and intraperitoneal dose of cyanamide

Dose* (mg kg^{-1})	Concentration of cyanamide		
	Plasma (ng ml^{-1})	Liver (ng g^{-1})	Mitochondria (ng g^{-1})
0·035	ND†	ND	ND
0·070	ND	ND	ND
0·175	ND	ND	ND
0·35	ND	ND	ND
0·7	12·5±4·3	ND	ND
3·5	44·3±8·5	63·5±12·7	ND
7·0	127·3±17·7	63·7±10·8	ND
14·0	274·2±43·3	192·2±43·2	205·8±38·5
56·0	3 192±420	1 358±239	1 488±237
112·0	5 938±1 459	4 494±1 239	4 323±1 156
224·0	15 520±3 342	14 470±1 125	9 259±2 688

*Total after seven equal administrations at 45-min intervals.
†Concentration non-detectable.

45 min dosing interval. Single doses ranged between 0·005 and 32 mg kg^{-1} (0·035–224 mg kg^{-1} total administered dose). The results obtained are shown in Table 1.

The repeated *intra*-peritoneal administration of cyanamide produced a dose-dependent inhibition of both low-K_m and high-K_m liver mitochondrial ALDH isozymes (Figure 1) showing saturation for the former and a progressive increase with dose for the latter. Differential sensitivity to the cyanamide-induced inhibition of both isozymes was observed with a DI_{50} value of 0·104 and 8·52 mg kg^{-1}, respectively. For the low-K_m isozyme, the inhibition was almost complete at 0·35 mg kg^{-1} total administered dose, while high-K_m ALDH

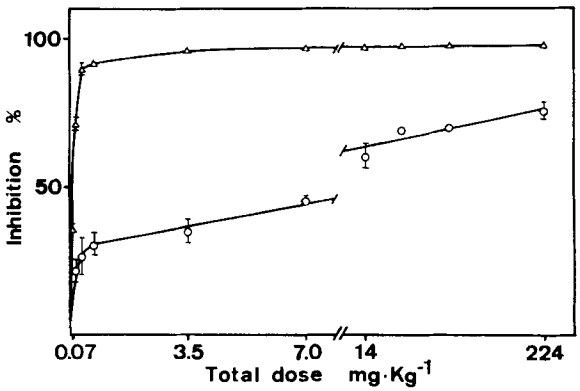

Figure 1. Inhibition of low-K_m (△) and high-K_m (○) hepatic mitochondrial ALDH activity vs administered dose of cyanamide. Data are expressed as mean±SEM ($n=5$)

Table 2. Mean (±SEM; $n=5$). Concentration–response curve data for the inhibition of high-K_m hepatic mitochondrial ALDH by cyanamide

Model	Compartment	I_{max}	IC_{50}	$r^{2\S}$	n¶	Schwarz criterion
I_{max} eq (1)	Plasma	74·75±2·89	68·64±20·64*	0·9347	-	−25·56
	Liver	75·45±3·17	53·74±13·55†	0·9263	-	−20·47
	Mitochondria	76·21±4·15	73·35±24·33‡	0·8611	-	−12·47
Sigmoid	Plasma	90·13±7·87	94·61±52·75*	0·9903	0·375	−17·72
I_{max} eq (2)	Liver	83·75±11·4	55·20±31·12†	0·9470	0·493	−19·92

*ng cyanamide ml^{-1} plasma.
†ng cyanamide g^{-1} liver.
‡ng cyanamide g^{-1} mitochondrial residue.
§Determination coefficient.
¶Hill coefficient.

inhibition was not complete after the highest total administered dose (80·7 per cent at 224 mg kg^{-1}).

The relationships between plasma, liver, and hepatic mitochondrial cyanamide concentrations, obtained at different doses of cyanamide (Table 1), and the inhibition of high-K_m ALDH (values from Figure 1) were analysed by means of I_{max} and sigmoid I_{max} models (see Materials and Methods). The results obtained after fitting equations (1) and (2) to the experimental data are summarized in Table 2. Application of the Schwarz criterion indicates that the sigmoid I_{max} model (equation (2)) describes the experimental data better than equation (1).

It has not been possible to determine if there is any relationship between plasma, liver, and hepatic mitochondrial cyanamide concentrations and the inhibition of the low-K_m ALDH. This is explained by the fact that, at the limit of quantitation of cyanamide in the three biological tissues, the inhibition of the enzyme was almost complete (see Figure 1).

The effect of dosing rate on the plasma concentration of cyanamide at apparent steady-state (C_{SS}) is shown in Figure 2. A greater than linear fractional increase in C_{SS} was observed over the dosing rate range studied, suggesting saturable metabolism of cyanamide for its elimination from the body or saturation of hepatic first-pass biotransformation during its absorption from the peritoneal cavity. In fact, equation (3) fits the experimental data of Figure 2 better than equation (4).

$$C_{SS} = \frac{K_m \cdot D_R}{V_m - D_R} \tag{3}$$

$$D_R = CL \cdot C_{SS} \tag{4}$$

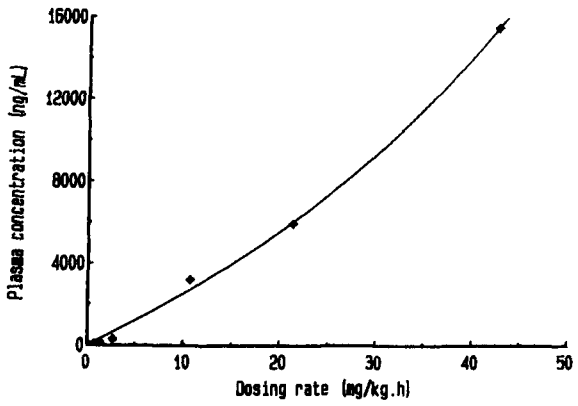

Figure 2. Effect of cyanamide dosing-rate on the plasma cyanamide concentration

where D_R is the dosing rate, CL is the total plasma clearance, K_m represents the plasma concentration at which half of the maximal rate of elimination is reached, and V_m is equal to the maximal rate of elimination.

DISCUSSION

The results described in Table 1 show a dose-dependent effect for plasma, total hepatic, and hepatic mitochondrial concentrations of cyanamide after repeated administration of this drug to the rat. The study was conducted under apparent steady-state conditions to minimize inter-individual variability due to distribution effects. The mitochondrial cyanamide concentration data indicate that cyanamide can cross the mitochondrial membranes. Thus, cyanamide itself could be available to the low-K_m ALDH isozyme, which is located in the mitochondria[28–30] to produce its inhibition. The possibility that cyanamide could be the true inhibitor of the enzyme has been pointed out in a previous study.[19]

Cyanamide can also reach the high-K_m mitochondrial ALDH isozyme which is bound to the outer mitochondrial membrane[28–30] and is different from the high-K_m cytosolic ALDH isozyme.

The hepatic mitochondrial low-K_m ALDH isozyme was more susceptible to cyanamide-induced inhibition than the high-K_m isozyme (Figure 1); the DI_{50} value for the low-K_m isozyme was 82-fold lower than for the high-K_m isozyme. The results agree with those published by Loomis and Brien[20] using calcium carbimide.

The relationship between high-K_m ALDH inhibition and plasma and liver cyanamide concentrations would be represented by a curve with a steeper slope at low concentrations and shallower at higher concentrations than the usual

quadratic hyperbola, typical of the I_{max} model. In fact, after application of the Schwarz criterion, the sigmoid I_{max} was selected as best representing the data; the steepness factor is then less than unity ($n = 0{\cdot}375$ and $n = 0{\cdot}493$ for plasma and liver, respectively; see Table 2). The sigmoid I_{max} model was not applied to the relationship between mitochondrial cyanamide concentration and the inhibition of the high-K_m isozyme because there were too few experimental data pairs in relation to the number of parameters in this model. The use of several models to determine the concentration–effect relationship has been discussed by Holdford and Sheiner.[31]

It has been pointed out that the effect of dosing rate on the plasma concentration of cyanamide at steady-state (C_{SS}) is described better by the non-linear equation (3) than the linear one, equation (4), indicating that clearance did not remain constant with increasing dose of cyanamide. In fact, clearance and elimination half-life determined in the rat after 2 mg kg^{-1} cyanamide administration were 177 ml kg^{-1} min^{-1} and 32 min, respectively;[21] the literature values are 20 ml kg^{-1} min^{-1} and 56 min, respectively, after 35 mg kg^{-1} cyanamide administration.[22] Non-linearity in these parameters with respect to concentration of drug could be related to saturation of plasma protein binding or hepatic biotransformation capacity. Saturation of hepatic first-pass biotransformation during absorption of cyanamide after intra-peritoneal administration is also a possibility. However, Shirota *et al.*[16] reported that cyanamide is rapidly and extensively metabolized to *N*-acetylcyanamide and excreted in urine by the rat, dog and rabbit. Non-linear pharmacokinetic behaviour of cyanamide, which is metabolized by the liver with a high extraction ratio, would be better explained by saturation of cyanamide biotransformation capacity than by saturation of protein binding capacity. In this situation, namely, when the clearance is concentration dependent, the equation $CL = V_m/K_m + C_{SS}$ based on the Michaelis-Menten model for enzyme kinetics could describe the non-linearity. Our results are in accord with the non-linear pharmacokinetic behaviour of cyanamide in dogs reported by Obach *et al.*[21]

In summary, the results described here showed that the low-K_m mitochondrial ALDH isozyme is notably more susceptible to cyanamide-induced inhibition than the high-K_m isozyme, as indicated by the DI_{50} values. Relationships between plasma and liver cyanamide concentrations and inhibition of high-K_m ALDH were better described by a sigmoid I_{max} model than by a I_{max} model. A non-linear relationship between cyanamide dosing rate and the plasma cyanamide concentration at apparent steady-state conditions was also defined.

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